SERUM PROFILING FOR THE DISCOVERY OF BIOMARKERS ASSOCIATED WITH LOW-LEVEL VAPOR EXPOSURE TO VX AND GB IN THE RAT AND MINIPIG MODEL

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1. ABSTRACT

The need to develop a rapid, low-level chemical nerve agent exposure detection system necessitates the development of a blood test that does not require individual initial baseline measurements. Toward this goal, we have begun a series of experiments to screen serum from animals exposed to low levels of OP nerve agents for biomarkers associated with exposure. This manuscript describes the use of strong anion exchange chromatography and Surface Enhanced Laser Desorbtion Ionization (SELDI)-Mass Spectrometry (MS) to efficiently screen serum for protein biomarkers of exposure to GB and VX in the minipig and rat models, respectively. Our most recent data indicates that several protein species are uniquely altered in the serum of low-level agent-exposed animals up to one week after exposure.

2. INTRODUCTION

While operating in a chemical or biological warfare environment, or potential terrorist incident, soldiers and first responders risk exposure to toxic substances that can result in operational deficits, incapacitating symptoms, or death. Although the mechanisms by which chemical weapons cause acute injury are well understood, personnel involved in decontaminating equipment or destroying chemical weapons, as well as persons on the periphery of an attack, may also face exposure to low levels that may not induce immediate symptoms. Soldiers face additional toxicological hazards from exposure to industrial chemicals and pesticides during deployment in areas of the world where environmental regulations are lacking. [1]

Scientists have been assessing the effects of chemical weapons on living systems for over 75 years. Most toxicological research up to this point, both inside and outside the U.S. Army, has consisted

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of chemical exposure of animals followed by observations of a limited number of physiological changes, including death.[2] This work has provided a valuable body of data that has been used to develop and establish personal protection, chemical detection and decontamination levels, and therapeutics for acute exposures.[3] However, exposures to low levels of nerve agent that do not result in immediate injury may potentially predispose soldiers to subtle operational deficits or to ailments that arise much later. Traditional toxicological methods lack the power to determine the mechanisms of action of toxic agents at the most fundamental level, that is, how genes and proteins ultimately determine the response to toxins, and how these changes manifest as pathology over time.[4]

Currently, determination of exposure to low levels of chemical nerve agents and other, more commonly encountered organophosphate (OP) compounds such as pesticides, is made by measurement of an individual's blood Acetylcholinesterase (AChE) level. This test relies upon relative comparison of the test results with a baseline AChE level. Unfortunately, there is significant individual variability in baseline AChE levels as well as in sensitivity of the enzyme level to low levels of OP exposure. Therefore, unless a baseline level has been recorded for an individual, it is nearly impossible to make a definitive measurement of OP exposure using this test. Given the reality that OP agents are present in the civilian sector (as pesticides), as well as in the hands of terrorist groups (i.e. Tokyo subway attack using Sarin), it is clear that a test to rapidly detect low-level exposure, without the need for baseline measurements, would greatly aid in the management of OP-agent related emergencies in the future.

The work described in this manuscript details our experiments using blood serum from rats and minipigs exposed to low levels of OP nerve agents (VX and GB, respectively) to screen for novel serum biomarkers associated with exposure. Through our collaboration with Ciphergen Biosystems, we have been able to rapidly assess the protein profiles of serum samples taken from the exposed and unexposed animals. Strong anion exchange chromatography was used to fractionate blood serum and increase the likelihood of detecting less abundant proteins. Surface Enhanced Laser Desorption Ionization (SELDI)-Mass Spectrometry (MS) rapidly and efficiently screened the fractions for protein biomarkers of exposure to GB and VX. Surface chemistries of the SELDI protein chips utilized in the experiments allowed the capture of a subset of proteins based on biochemical characteristics. This preliminary data, indicating the persistence of a measurable serum response, suggests a potential use of this data to develop a rapid, screening test for low level exposure to nerve agents . Such as test would potentially have at least two applications: 1) could reveal physiological evidence of immediate exposure resulting from terrorist attacks, warfare, and industrial accidents and 2) could reveal longer-term (one week, perhaps longer?) post-exposure evidence that individuals suspected of illegally manufacturing or handling chemical nerve agents had been exposed to low levels of nerve agent.

3. BACKGROUND

3.1 USE OF SELDI TO SCREEN FOR BIOMARKERS

Proteins, polypeptides, and peptides comprise the greater part of the body framework as well as serve as the work horses of physiological function and communication. Over 60% of blood consists of serum, which contains water, electrolytes, albumin and other important proteins. Changes in the relative composition of serum protein mixtures or characteristics of individual serum proteins can indicate response to specific events such as drug exposure, diet, dehydration, stress, and temperature.[5] Infectious disease, degenerative disorders, cancer and immune dysfunction all result in changes in protein composition and in the occurrence of new or altered proteins. Since the blood flushes through all of the body's organs, it is an ideal reservoir of proteins in which to seek key proteins that may be a sign or signal of dysfunction, injury, or disease.[6] Such protein biomarkers can provide a sentinel warning much earlier than the appearance of physiological symptoms.

The benefits of protein arrays are tremendous. Surface chemistries of the ProteinChip® allow capture of subsets of proteins based on biochemical characteristics that can distinguish differences in

protein binding among samples.[7] Use of protein arrays reduces complexity of protein composition and allows for greater analysis of the proteome. A high-throughput SELDI-MS system such as Ciphergen's PBS IIC machine (**Figure 1**) ionizes the protein samples with a laser; fragments are read and then analyzed by the detector that feeds information to the ProteinChip® software. Bioinformatics and Biostatistical tools, such as those designed by Ciphergen, can facilitate clustering analysis of data.

Correlation of the protein array data with information from gene array data (not discussed here) of same sample set combined with bioinformatics and biostatistical tools will allow information from both crucial studies to be integrated to form a more complete picture of molecular response to agent exposure.

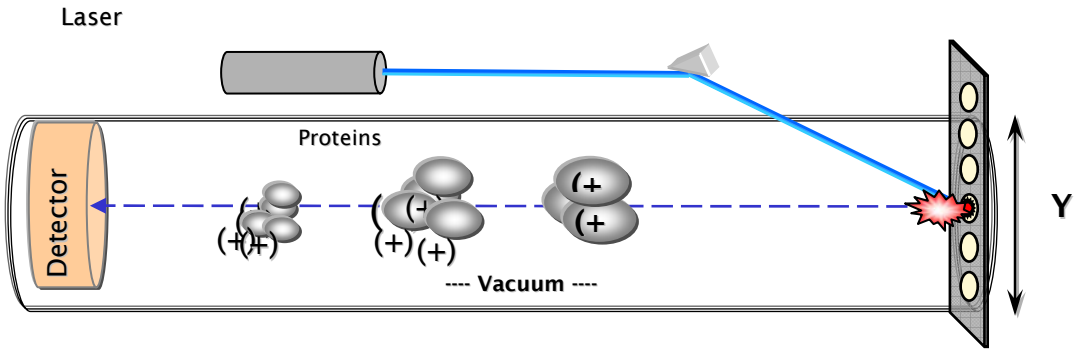

Figure 1. Diagram of SELDI ProteinChip® array reader. Protein samples are ionized by the laser and fragments are read and analyzed by the detector that feeds information to the ProteinChip® software.

3.2 SARIN AGENT[8]

Sarin (GB, Military designation) is a colorless, relatively odorless organophosphate (OP) nerve agent. The substituent groups surrounding the pentavelent phosphorous atom dictate its properties. A volatile liquid at room temperature, it has a high absorption rate in most fabrics along with a high desorbtion rate. It hydrolyzes readily at pHs less than 4 and greater than 7 and creates hydrogen fluoride (HF) and methyl phosphonic acid spontaneously.

3.3 VX AGENT[9]

VX is a colorless, mostly odorless, and oily liquid organophosphate (OP) nerve agent. It is the least volatile and most persistent of all known nerve agents in addition to being extremely lipid soluble. Being the most toxic of all known nerve agents, one drop of the compound is fatal compared to 1-10 milliliters of any of the G agents. Due to its persistence, contamination of equipment and terrain lasts for days.

3.4 KNOWN EFFECTS AND MECHANISMS OF ACTION OF SARIN (GB) AND VX

Extensive toxicological research has been conducted for acute exposure (less than 24 hours) to the G agents and VX and is extremely well documented.[10] Many chronic exposure studies in animals illustrate cholinergic and non-cholinergic effects, EEG changes, behavioral and psychological effects, and other neurotoxicity such as organophosphate induced delayed neuropathy (OPIDN). No teratogenicity/reproductive effects have been found due to acute exposure to GB, nor has any carcinogenecity, genotoxicity, or cardiomyopathy been found.[11]

The primary mechanism of action for GB and VX are similar (see **Figure 2**). The agents irreversibly bind to Acetylcholinesterase (AChE) causing its inhibition and inability to bind to Acetylcholine receptors in the central nervous system.[12] This results in accumulation of Acetylcholine at the synapses and continued firing of the neurons ultimately resulting in death.

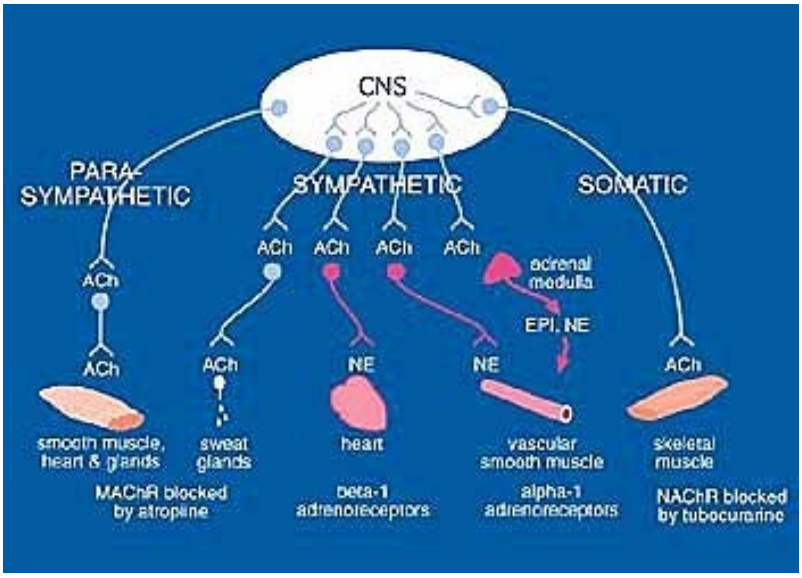

Figure 2. Schematic of cholinergic response.

Progression of symptoms due to exposure varies with the dose of agent, which also depends on the level, duration, and route of exposure. Low exposure has revealed dimness of vision (miosis), runny nose, tightness of chest, and tearing. Medium doses include those symptoms present in low doses with the addition of sweating, eye pain, headache, stomach cramps, nausea, vomiting, forgetfulness, irritability, depression, and changes in heart rate. High doses are indicative of low and medium dose symptoms along with loss of muscle control, twitching, convulsions, paralysis, unconsciousness, coma and eventually death.[13]

4. EXPERIMENTAL METHODS

4.1 GB VAPOR GENERATION

Sarin (GB) samples (98.16+/-0.36% purity by NMR) were obtained from the U.S. Army Edgewood Chemical Biological Center. A saturator cell produced GB vapor that was drawn through a 1000-liter dynamic airflow inhalation chamber constructed of stainless steel with Plexiglas windows.

4.2 MINIPIG EXPOSURES AND PHYSIOLOGICAL MONITORING

Ellegaard Gottingen Minipigs (5-6 months old from Marshall Farms) were strapped into stainless steel and cloth slings. Male and female pigs were exposed, via whole body inhalation, to a fixed concentration of GB vapor (varying with each pig) for 10, 60, or 180 minutes. Sub-lethality clinical signs, such as miosis, were monitored during and after exposure. Blood Cholinesterase (AChE and BuChE) activity was measured (modified Ellman Method) from pre-exposure and post-exposure blood samples taken from a catheter surgically inserted in the neck. The effect of GB exposure on pupil size

(diameter) was assessed pre- and post-exposure using an infrared (IR) digital camera (Data Science Automation) under low light conditions (<10 candles).

4.3 VX VAPOR GENERATION

VX samples (93.6+/-0.5% purity by NMR) were obtained from the U.S. Army Edgewood Chemical Biological Center. A saturator cell system produced VX vapor that was drawn through a 750-liter dynamic airflow inhalation chamber constructed of stainless steel with Plexiglas windows.

4.4 RAT EXPOSURES AND PHYSIOLOGICAL MONITORING

Sprague-Dawley rats (7-8 weeks old from Charles River Laboratories) were confined in stainless steel compartmentalized cages (20" x 14"x 4") with each rat housed in a separate compartment. Male and female rats were exposed, via whole body inhalation, to a fixed concentration of VX vapor (0.000374 [female only], 0.00061 [male only], 0.00067, 0.00105, or 0.00183 mg/m3) for 240 minutes. Lethality and sub-lethality clinical signs, such as miosis, tremors, salivation, lacrimation, labored breathing, convulsions, were monitored during and after exposure, although miosis was the only observable sign noted. Blood cholinesterase (AChE and BuChE) activity was measured (modified Ellman method) from pre- and post-exposure blood samples taken from the tail vein. The effect of VX exposure on pupil size (diameter) was assessed pre- and post-exposure using an infrared (IR) digital camera (Data Science Automation) under low light conditions (<10 foot candles).

4.5 SERUM EXTRACTION

Minipig blood was drawn through a catheter inserted into a major vein the neck during an anesthetized surgical procedure. Blood samples were collected immediately pre-exposure, immediately post-exposure, and prior to necropsy (approximately 3 ml each). Blood was briefly kept on ice and fractionated in a swinging bucket microcentrifuge at 4000 rpm for 5 minutes. Serum was drawn off, aliquoted, flash-frozen in liquid nitrogen, and stored at –80 º C until analysis. Rat blood was drawn from the tail tip into small BD microtainer tubes (approximately 1 ml) prior to organ harvest (7 days post-exposure) and placed on ice. Blood samples were briefly kept on ice and then fractionated in a fixed-angle microcentrifuge at 16,000 rpm for 5 minutes. Serum was drawn off, aliquoted, flash-frozen in liquid nitrogen, and stored at –80ºC until analysis.

4.6 PROTEIN PROCESSING AND HYBRIDIZATION TO PROTEINCHIP® ARRAYS[14,15]

Microscale serum fractionation was performed by initially denaturing samples with 9M urea/2% CHAPS buffer. The denatured samples were applied to Q HyperD resin in 96-well plates for anion exchange chromatography. Six fractions were collected per sample. Minipig fractions (1, 3, and 4) were applied to two types of ProteinChip® arrays, CM10 and IMAC30-CU arrays. Rat fractions (1 and 4) were profiled on IMAC30-CU, H50, CM10, and Q10 arrays.

IMAC30 arrays are charged with Cu^{2+} and PBS, using a buffer of pH 7/0.5M NaCl for binding and washing. H50 hydrophobic arrays are pre-activated by a bulk wash in 50% acetonitrile (ACN) and dried; a buffer of 10% ACN/0.1% TFA is used for binding and washing. CM10 weak cation exchange arrays have 50mM NaOAc/pH4 buffer used for binding and washing. Q10 strong anion exchange arrays use a buffer of 50 mM Tris/pH9 for binding and washing.

All arrays are washed and air dried, then spotted 2 times with 50% SPA, prepared in 50% ACN/0.5% TFA. Both pig and rat sample chips were read by the ProteinChip® AutoBiomarker system on the Ciphergen PBS IIC ProteinChip® Reader and data was analyzed using the ProteinChip® software and CiphergenExpress™.

4.7 PREPARATION OF DATA[16,17]

Data collection from the ProteinChip® arrays was conducted with an autoloader on the Ciphergen PBSIIC ProteinChip® Reader allowing for collection without manual intervention. After collection, the baseline was subtracted and data was normalized based on total ion current. Peaks were chosen using the Biomarker Wizard tool in the ProteinChip® software.

4.8 ANALYSIS OF PEAK DATA[18,19]

Peak information was exported from ProteinChip® software for analysis using GraphPad Prism® software. Peak intensities of replicate minipig samples were averaged for accurate p-value determination. Univarient nonparametric analysis (ANOVA) compared all 3 groups and all three combinations of paired groups (Bonferroni's Multiple Comparison Test). The aim of this analysis was to find peaks that distinguish the samples based on the 3 time points for pigs: Pre-exposure (group 0), immediately after exposure (group 1) and necropsy (group 2). For rats, statistical tests such as the Bonferroni's Multiple Comparison Test, Kruskal-Wallis test, and calculation of p-value were used. Biomarkers were selected based on discrimination of exposed samples from control samples.

5. RESULTS AND DISCUSSION

Since the description of Gulf War Syndrome, there has been increased interest in the effects of sub-acute and low-level exposure to chemical agents, including Organophosphate (OP) compounds.[20] While the acute and overt effects of OP agent toxicity have been extensively studied, the subtle and molecular level alterations that occur in response to these agents are not well understood. Agent-induced damage and alterations occurring at the molecular level have been implicated in the etiology of injury and disease that may present itself in weeks, months, and even years after the initial toxicant exposure.[21]

5.1 VX RAT STUDY[22]

Serum samples from 10 female rats exposed to a VX dose of 0.00067 mg/m^3 for 240 minutes (0.1608 $mg*min/m^3$) by full-body inhalation, and 5 female rats exposed as air controls for 240 minutes were analyzed. Seven of the 10 exposed female rats demonstrated miosis (pupillary constriction) at 1 hour post exposure. Protein arrays used were IMAC30, H50, CM10, and Q10. Data for the VX rat study indicates a difference in the exposed animals that did not exhibit miosis from the control rats and those rats with miosis (**Figures 3-5**). The peaks with the lowest p values indicate the potential biomarkers which discriminate exposed samples without miosis from control samples. This may predict which rats are not sensitive to the level of nerve agent tested.

To the extent of the study completed so far (fraction 1, 4 surfaces; fraction 4, 2 surfaces), there are no serum proteins differentially expressed between exposed with miosis and control rats. This may be a result of the time of blood draw - 1 week post exposure (recovered miosis observed in all animals by 36 hours). Sampling blood immediately following exposure and various time points thereafter may result in the discovery of differential protein expression between control and exposed rats. A closer look at additional fractions, arrays, and binding/washing conditions may result in the discovery of proteins that differentiate exposed and control rats.

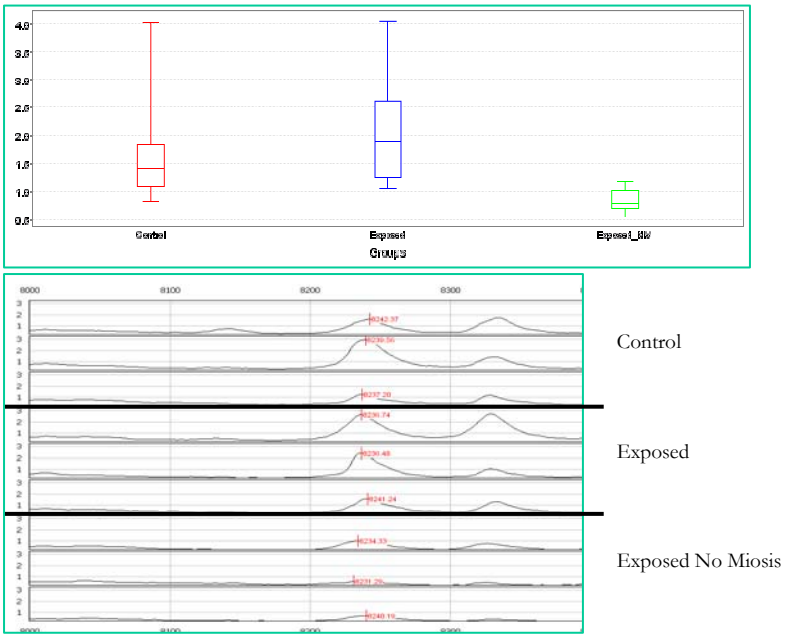

Figure 3. CM10, fraction 1, low mass: 8.2 kDa differentially expressed protein ($p<0.032$)

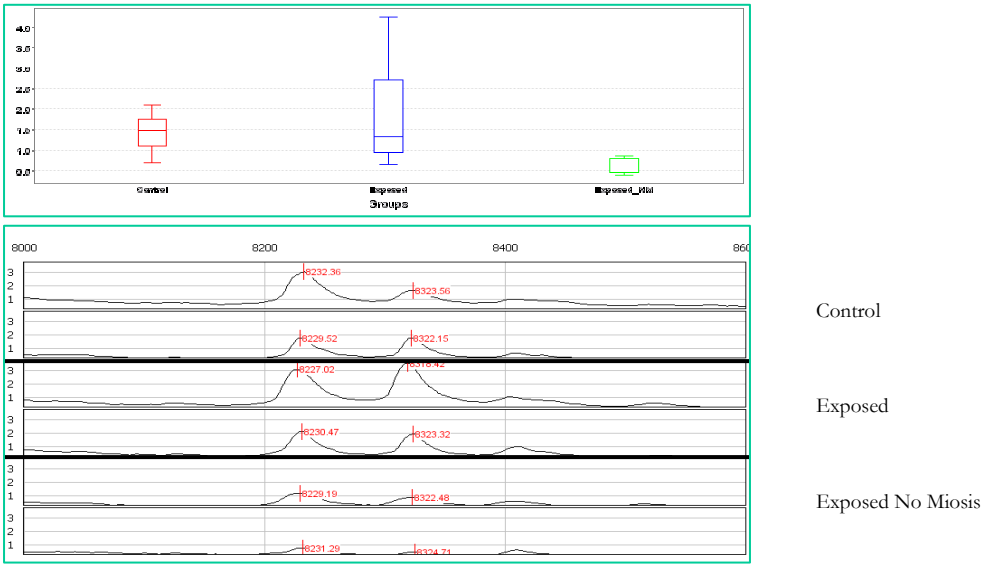

Figure 4. CM10, fraction 1, low mass: 8.3 kDa differentially expressed protein ($p<0.049$)

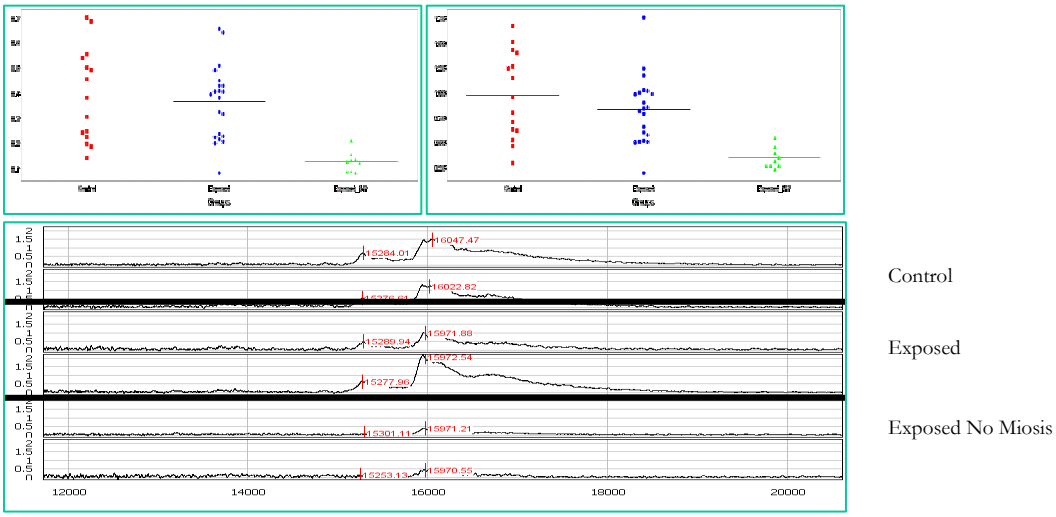

Figure 5. H50, fraction 1, low mass 15.3 kDa and 16.0 differentially expressed proteins (p<0.026, 0.032, respectively)

5.2 GB MINIPIG STUDY[23]

Three serum samples from each of 7 female pigs prior to and post low-level GB exposure were analyzed. Group 0 in the figures indicates immediately pre-exposure, Group 1 indicates immediately post-exposure, and Group 2 indicates necropsy. All pigs received different doses of the Sarin. CM and IMAC-Cu ProteinChip® arrays were utilized in this portion of the study.

Using the ANOVA analysis results comparing all three groups and identifying peaks that separate time points, the data for the minipig study indicates 15 candidate markers with p =or< 0.05 for early onset transient, early onset sustained, and late onset markers. While peak intensities differ, the relative profiles of the potential markers are consistent between animals. Figure part A displays the overall expression pattern for this potential marker and B is the pattern for each animal. C is the highlighted raw data from each sample.

When possible, classification of the timing off a biomarker change (either increase or decrease) will add to the understanding of the biology at work in the system under study. This timing can also impact the utility of a given biomarker as a diagnostic for a biological response. For studies of short duration, such as this one, three classes are commonly defined. In this study, peak intensity changes that appear in most of the animals only at the 24 hour time point (seen in group 2 data) are *late onset*; if they appear immediately after exposure and remain at the 24 hour time point (group 1 and 2) they are *early onset sustained*; and if they appear immediately after exposure then return to normal levels by 24 hours (group 1) they are *early onset transient*.

In **Figure 6**, results from a protein at 8880 Da from fraction 1 captured on IMAC30-Cu ProteinChip® Array are shown. This marker displays a transient increase in peak intensity in most animals, but return to pre-exposure levels by 24 hours. The statistical results are as follows: ANOVA Repeated Measures p=0.0162, Bonferroni's Multiple Comparison Test p<0.05 (0 vs 1).

Figure 7 illustrates results from a protein at 16045 Da from fraction 4 captured on the IMAC30-Cu ProteinChip® Array. This marker displays a decrease in peak intensity in most animals 24 hours after initial exposure to Sarin. The statistical results are as follows: ANOVA Repeated Measures p = 0.0064, Bonferroni's Multiple Comparison Test p<0.05 (0 vs 2, 1 vs 2).

Figure 8 indicates results from a protein at 39583 Da from fraction 3 captured on IMAC30-Cu ProteinChip® Array. This marker displays a sustained increase in peak intensity in most animals. The statistical results are as follows: ANOVA Repeated Measures p=0.0366, Bonferroni's Multiple

Comparison Test $p < 0.05$ (0 vs 1). This candidate biomarker has lower significance values, and when looking at the bar graph it is clear that the assignment of this biomarker to the early sustained class is tentative. The increase in the biomarker is also not seen in as many animals due to individual animal differences. This phenomenon is common and is the driving force behind the use of multivarient analysis methods.

Thus far, the results of this pilot study demonstrate the utility of the protein array technology to identify alterations in protein expression induced by OP agents. Furthermore, collection of data at lower doses of OP agents and at time points closer to and further from the initial dose point will be required to begin to build a more complete understanding of lower-dose and exposure duration to OP agents.

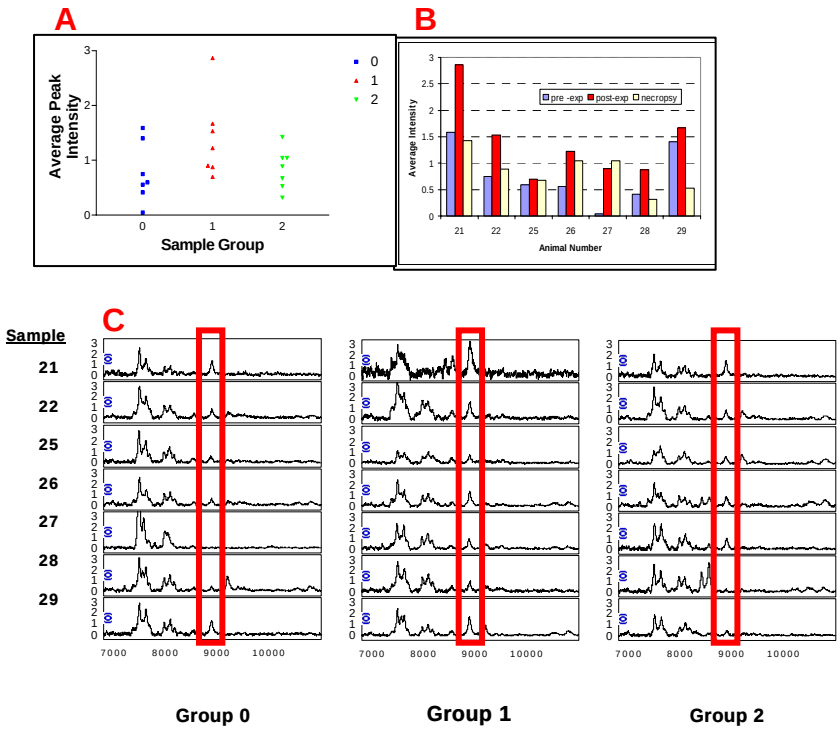

Figure 6. Early transient marker, increased immediately post-exposure. Observed 8880 dalton peak in fraction 1 on IMAC surface.

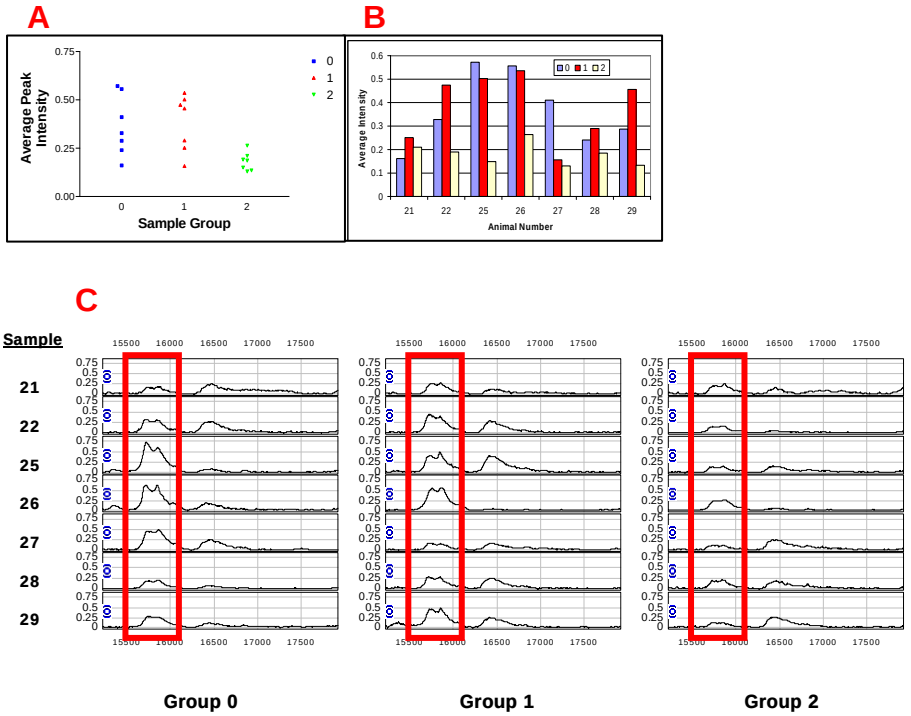

Figure 7. Late onset marker, decreased post-exposure. Observed 16045 dalton peak in fraction 4 on IMAC surface.

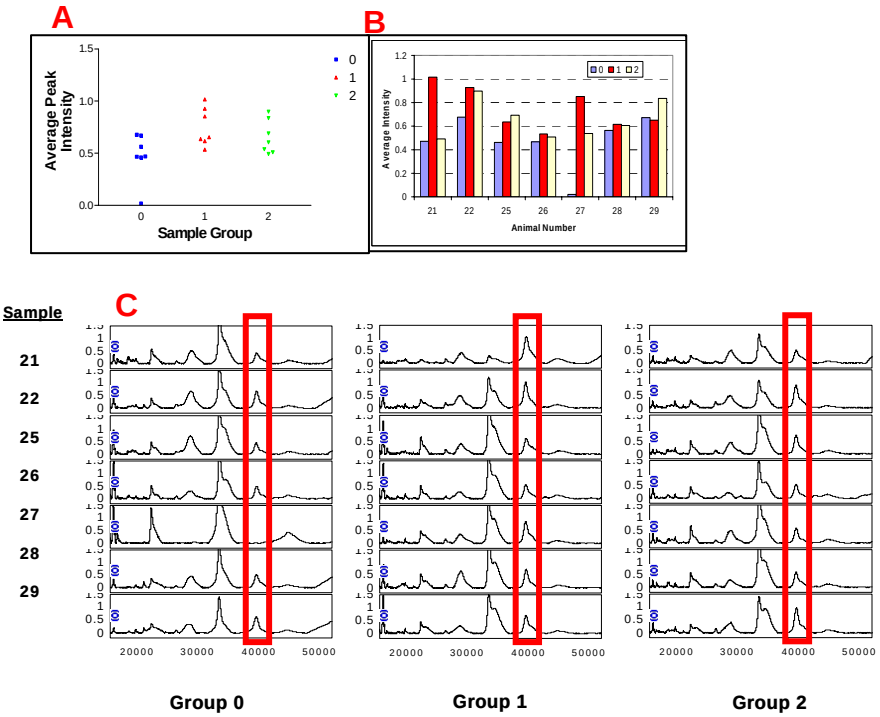

Figure 8. Sustained early onset marker, increased post-exposure. Observed 39583 dalton peak in fraction 3 on IMAC surface.

6. CONCLUSIONS AND FUTURE DIRECTIONS

Thus far, the results of this initial study demonstrate that there are several protein species altered through exposure to low-level doses of VX and GB. Exposure of the female rats to VX vapor at 0.1608 mg*min/m^3 (0.00067 mg/m^3 for 240 minutes) results in serum proteins expressed which differentiate exposed rats without miosis from exposed rats with miosis and controls. Expression of proteins in serum may protect or predict those rats that are not sensitive to the level of nerve agent exposure tested. Using these two fractions (1 and 4) from anion exchange chromatography, there are no serum proteins that are differentially expressed between exposed and control rats. Examining blood taken immediately following exposure and at time points earlier or later than one week post exposure may result in discovery of differential protein expression between exposed and control rats.[24]

Exposure of the female pigs to GB vapor at various levels for 10, 60, or 180 minutes results in 15 candidate markers with $p =or< 0.05$ (ANOVA), which can be divided into two classes: early transient, sustained early, and late onset markers. While most animals follow a pattern of marker expression others do not, making some of these markers more convincing when considering the results of individual animals. A larger sample set size and more replicates for each dose group would add statistical significance to these preliminary data.[25]

The classification of all these candidate biomarkers as early or late onset isn't absolute. When looking at the data from individual animals in some cases, another classification could also fit. It is rare to find statistically significant biomarkers that show 100% correlation with the sample group divisions, and in some cases the correlation is moderate at best. This type of variability among individuals is typical and seen with all the candidate markers found in this study. Additionally, since genetic and environmental variability significantly affect the protein profiling results generated with all methods, the use of multivarient analysis tools to increase the confidence of assigning unknown animals to a particular group is highly encouraged.[26]

Expanding the sample set size to increase the number of statistically significant differences among the groups in rats and pigs size will add statistical power to these preliminary data. Examination of potential protein biomarkers in tissue such as brain and liver in addition to blood serum from minipigs exposed to GB and rats exposed to VX should add confirmation of changes in the body proteins. An increase in the number time points of sampled blood for rats to before exposure and immediately after exposure in addition to one week post will show more promise in finding additional biomarkers that discriminate exposed samples from controls. Investigation of additional anion exchange chromatography fractions, arrays, and binding/washing conditions will cover a wider range of proteins. With the addition of more data in the near future, we hope to identify protein expression alterations unique to each agent or common to these and other OP agents.

7. ACKNOWLEDGEMENTS

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